

ground due to oxygen. This background ECL however, does not prevent one from obtaining peroxide detection limits below 1  $\mu\text{M}$ . The ECL intensity varies with both pH and potential. The net ECL to background ECL ratio is a maximum at pH 10. Gold and glassy carbon electrodes are preferable to platinum, but gold is somewhat better than glassy carbon. Potentials more negative than +0.3 V yield insignificant ECL; potentials of +0.4 to +0.6 V yield maximum ECL intensity. Long-term stability of the ECL signal is improved by use of a potential waveform which alternates between a positive potential where luminol is oxidized and a negative potential (about -0.2 V) where the electrode surface is reduced.

If luminol or luminol-labeled species are the analyte, then one needs both peroxide and catalyst as the reagents. Because the catalyst can be achieved electrochemically, it makes sense also to consider electrochemical generation of the peroxide from oxygen and water. In a stream flowing through an electrochemical flow cell, as long as the oxygen concentration, pH, flow rate, and electrode behavior remain constant, the amount of peroxide produced at the electrode per unit time will remain constant so that a constant concentration of peroxide will be achieved in the flowing stream. Use of a glassy carbon generating electrode, poised at about -1.0 V, yields sufficiently high peroxide concentrations over the pH 9.5 to 11 range to be useful with luminol CL; those solution pH values are also compatible with the luminol CL reaction. By using two electrodes in series (an upstream electrode to generate peroxide and a downstream electrode for luminol ECL) we have determined luminol from 0.1 nM to 10  $\mu\text{M}$ .

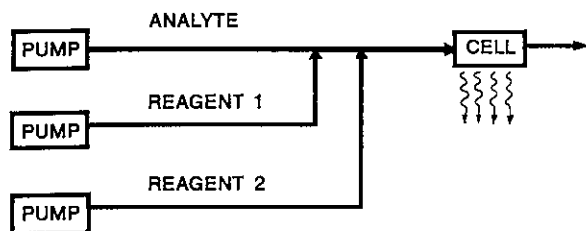


Figure 1. Typical chemiluminescence flow system using dissolved reagents.

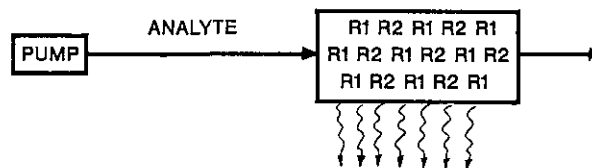


Figure 2. Possible chemiluminescence flow system with immobilized reagents (R1 and R2).

## *Applications of Lasers in Bioanalytical Chemistry*

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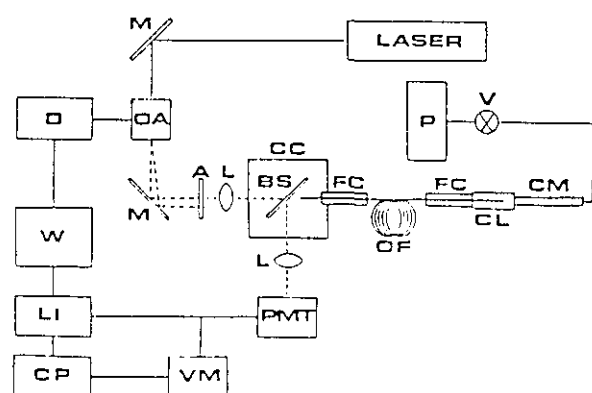
The combination of laser detectors and liquid chromatography (LC) has led to new types of analytical measurements in solution. LC has benefited from these selective detectors because, for complex biological matrices, complete separation of the analytes is rarely possible. Examples are polarimetry for distinguishing biologically important species from the bulk biological fluids and two-photon excited fluorescence. Laser spectroscopy has also benefited from the sample clean-up step provided by the LC, and the fact that the LC baseline allows a convenient reference measurement.

Absorption is a difficult spectroscopic measurement in remote monitoring situations. A well defined pathlength must be present to avoid nonlinear response. Unlike fluorescence, the measured radiation is at the same wavelength as the incident radiation, and scattered light is a problem. It is possible to use two optical fibers to direct light in and then out of the sample area, at the expense of a larger probe volume and potential cross-talk between fibers. When one fiber is used, reflections and scattering from the optics must be overcome. We use index-matching fluids to compensate for reflections at the end faces of the fiber. By modulating the laser source at a high frequency, the phase difference of the signals at the entrance of the fiber and at the remote optical region can be adjusted to 90°. Then, a lock-in amplifier can be used to distinguish

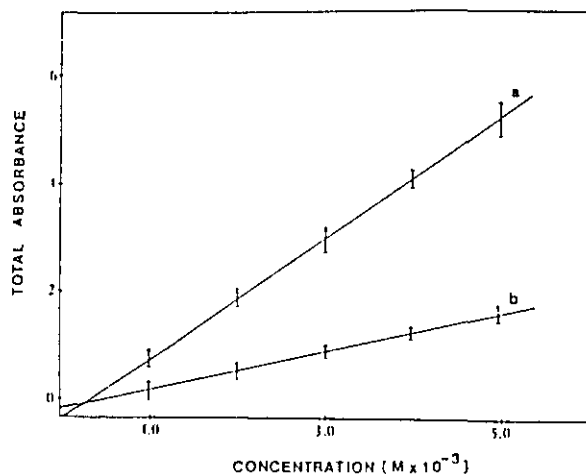
*Accuracy in Trace Analysis*

the two, leading to accurate absorbance values (figs. 1 and 2). We used this system for remote absorption detection of the effluent from a microbore LC column, and good detection capabilities were obtained.

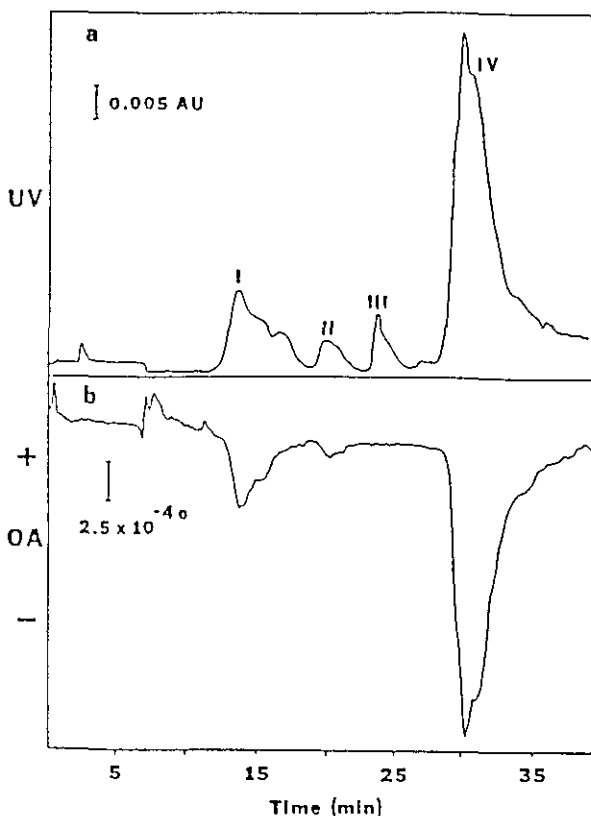
In the last decade, the use of high performance liquid chromatography (HPLC) for protein separation and purification has increased dramatically. Reversed-phase (RP) separations are popular because of the high resolving power available and widespread use of RP-HPLC in many other areas. But the conditions which produce good separations are known to significantly change the protein structure. Broadened or multiple peaks resulting from subjecting a single protein to RP-HPLC have been observed by several researchers for a variety of proteins. The HPLC of soybean trypsin inhibitor was reexamined by using simultaneous optical activity and ultraviolet absorption detection (fig. 3). Ratio plots of the two detector responses allow easy identification of impurities that were not related to the protein (fig. 4). The specific rotations of each of the separated components can be derived. We find that one denatured form has a distinctly lower specific rotation while another form shows no change in specific rotation. The on-column denaturation rate here was found to be slower than that from previous work. Column pretreatment may have resulted in milder column conditions through the elimination of irreversibly adsorbing sites.



**Figure 1.** Fiber optic absorbance probe and chromatographic system: P, pump; V, injection valve; CM, microbore column; CL, absorbance cell; FC, fiber chuck; OF, optical fiber; CC, coupling cell; BS, beam splitter; L, lens; A, aperture; M, mirror; OA, Bragg cell; Laser, HeNe laser; D, driver; W, square-wave generator; LI, lock-in amplifier; CP, computer; VM voltmeter; PMT, photomultiplier tube.



**Figure 2.** Beer's law plots of (a) modulated and (b) nonmodulated bromocresol green absorbance signals. Plots represent 95% confidence intervals for three replicate measurements.



**Figure 3.** Ultraviolet absorbance (a) and optical activity (b) chromatograms of soybean trypsin inhibitor. 115 µg injected.

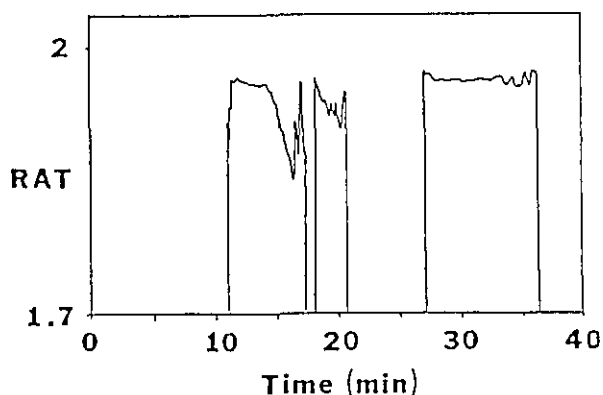


Figure 4. Plot of ratio (RAT) values versus time for the chromatograms shown in figure 3.

### *Trace Biogenic Amine Analysis with Pre-Column Derivatization and with Fluorescent and Chemiluminescent Detection in HPLC*

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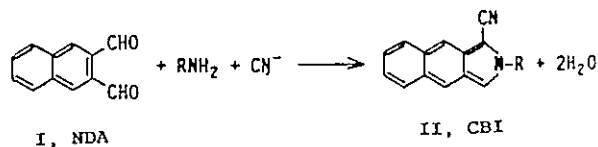
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#### **Background**

Research at the Center for Bioanalytical Research (CBAR) is aimed toward the development of highly sensitive and selective methods for the analysis of biological substances. The late Professor Takeru Higuchi, in creating the Center in 1983, initially focussed on target analytes such as amines, amino acids, peptides and polypeptides. Although methods such as RIA provide sensitive and selective analysis, a decision was made to develop fluorescence, chemiluminescence and electrochemistry for detection and liquid chromatography for separation; i.e. selectivity. Since most amino acids and peptides are optically and electrochemically "silent," it is necessary to derivatize these substances so that they can be detected. The objective of the present work was to develop a derivatization

scheme with LC separation and with fluorescence (FL) and chemiluminescence (CL) detection of catecholamines and other biogenic amines in biological samples.

It is well known that electrochemical (EC) methods offer high sensitivity detection for LC analysis of catecholamines in biological samples [1-5]. Luminescence detection methods, such as FL and CL, are also capable of providing high sensitivity detection [6-9], particularly when the weakly fluorescing amines are fluorescently labelled [8-14] by derivatization. Fluorogenic reagents such as ortho-phthalaldehyde (OPA) and fluorescamine (FCA), which are specific for primary amines [15,16], have been used to enhance the sensitivity of catecholamines for LC analyses. The OPA method has been significantly improved by CBAR with the development of a new fluorogenic reagent, 2,3-naphthalenecarboxaldehyde (**I**, NDA), which in the presence of cyanide ion, reacts with primary amines to give the intensely fluorescent product, 1-cyano-2-substituted-benz[f]isoindoles (**II**, CBI) [18].



The stability and FL quantum yields of the CBI-amine derivatives were superior to those from the OPA-thiol reaction [18]. The reaction of NDA with dopamine (DA), norepinephrine (NE), and the trace amines, octopamine (OA) and tyramine (TA), as a pre-column fluorogenic LC derivatization method, for the simultaneous determination of DA and NE in urine has been studied.

#### **Experimental**

**Instrumentation:** Optical absorbance, fluorescence and chemiluminescence measurements were made with a Shimadzu Model UV-260 spectrophotometer, a Farrand System 3 scanning spectrofluorometer, and an ATTO Model AC220 luminometer, respectively. The isocratic LC system consisted of a LKB Model 2150 dual-piston pump equipped with a Rheodyne 7125 sample injector with a 5  $\mu\text{L}$  sample loop. For some of the LCCL studies, an ISCO Model 314 syringe pump was used.